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Running title: VEGF and its receptors in renal cell carcinoma

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ABSTRACT

Background. Tumor angiogenesis is a dynamic process that plays a major role in cancer progression. Vascular endothelial growth factor (VEGF) and its receptors play a pivotal role in angiogenesis. The authors investigated the expression of VEGF and its receptors VEGFR-1 and VEGFR-2 in renal cell carcinoma (RCC) in the perspective of anti-VEGF treatments.

Methods. Total VEGF protein levels were quantified by ELISA in tumor tissue samples from surgical specimens of 65 patients with clear cell RCC. At the cellular level VEGF isoforms, VEGFR-1 and VEGFR-2 mRNA were quantified by real time quantitative RT-PCR in laser microdissected tumoral epithelial as stromal cells and in corresponding normal tissue compartments. Colocalization of VEGF and VEGFR-1 proteins was studied by triple immunofluorescent labeling.

Results. Protein VEGF in cytosolic extracts was significantly higher in tumoral than in non tumoral tissue ($p < 0.0001$). Event free survival was significantly longer for patients with cytosolic VEGF lower than the cut-off (75th percentile of VEGF protein levels, $p = 0.02$). In laser-microdissected epithelial cells, $VEGF_{121}$ and VEGFR-1 mRNA expressions were higher in RCC than in corresponding non tumoral kidney ($p = 0.007$ and $p = 0.002$, respectively); they were also higher in stromal cells of RCC compared with non- tumoral kidney ($p = 0.02$ and $p = 0.003$, respectively). There was no differential VEGFR-2 expression in epithelial or in stromal cells of tumoral or non-tumoral kidney. By immunofluorescent labelling VEGF and VEGFR-1 colocalized on RCC tumor epithelial and stromal cells.

Conclusion. Combined laser microdissection and quantitative RT-PCR, as triple immunofluorescent labelling, underlined the preferential expression of the most soluble VEGF isoform, $VEGF_{121}$, and its receptor VEGFR-1, but not VEGFR-2, in epithelial and stromal cells of RCC.

Keywords: Laser microdissection, renal cell carcinoma, VEGF, VEGFR-1, VEGFR-2.

INTRODUCTION

Tumor angiogenesis is a dynamic process that plays a major role in cancer progression, particularly in renal cell carcinoma (RCC) (1-3). Vascular endothelial growth factor (VEGF) and its receptors play a pivotal role in physiological and pathological angiogenesis. Therapeutic molecules directed against VEGF protein and VEGF receptor signalling are now available for RCC treatment (3, 4). Therefore, further characterization of VEGFR expression on RCC cells and investigation into VEGF as a growth factor in RCC is needed.

VEGF binds with high affinity to the receptor tyrosine kinase VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1, KDR), which upon ligand binding become tyrosine phosphorylated and activate multiple signalling networks (5). VEGF increases microvascular permeability, induces endothelial cell proliferation, survival, migration and differentiation, promotes the degradation of the extracellular matrix around the sprouting endothelium by inducing the expression of proteases. VEGF has five main isoforms produced by alternative splicing of a single gene located on 6p21.3, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{145}$ and $VEGF_{206}$ (6, 7). These isoforms differ primarily in their bioavailability, which is conferred by heparin and heparan-sulfate binding domains encoded by exons 6 and 7. $VEGF_{189}$, $VEGF_{145}$ and $VEGF_{206}$ contain additional stretches of basic residues, resulting in their nearly complete retention in the extracellular matrix. $VEGF_{165}$ exists both as an extracellular matrix-bound and as a soluble form released by proteolysis (2, 8). $VEGF_{121}$, which lacks both exon 6 and exon 7, is a highly diffusible protein. Several studies point out $VEGF_{121}$ and $VEGF_{165}$ as the most expressed isoforms in human tissues and tumors (9-11). Tumor progression involves complex interactions between neoplastic epithelial cells and the surrounding stroma composed of fibroblasts, endothelial cells, smooth muscle cells, inflammatory cells, and of a macromolecular network termed the extracellular matrix. In organs, tissue homeostasis is dictated by interactions between stromal and epithelial cells. During pathological processes, particularly cancer, both the epithelial and the stromal compartments undergo crucial changes for tumor progression (12). Supportive functions, such as angiogenesis, are provided by the stromal

components and allow tumor growth. Both stromal and epithelial cells are able to produce VEGF, and the expression of VEGF receptors R-1 and R-2 by epithelial cells from normal and neoplastic tissue has been reported (2, 13).

In the present study, we used laser microdissection coupled to real time quantitative PCR (QRT-PCR) to assess the respective expression of VEGF and its receptors transcripts in RCC tumor and stromal cells. At the protein level we analyzed VEGF quantitative expression in tissue extracts and VEGF and VEGFR-1 distribution and coexpression in the different types of cells.

PATIENTS AND METHODS

Patients

Between January 1998 and May 2001, 100 patients with untreated RCC were admitted to the department of urology of Hôpital Saint-Louis (Paris, France). RCC was histologically subclassified according to the current classification of adult renal epithelial neoplasms (14). Sixty-five patients with conventional cell renal carcinoma were included in this study (47 men, 18 women; median age 65 years: 31-88) (Table 1). Among the 35 RCC samples excluded, 16 were papillary carcinomas, 5 were chromophobe carcinomas and 9 were conventional RCC in patients for whom no follow-up was possible. All patients consented to this study. Before surgery, all patients underwent clinical examination and thoracoabdominal scan. Staging was done according to the TNM classification (15). T and N were always defined as pT and pN. The 65 patients underwent nephrectomies (radical, n = 59; partial, n = 2; tumorectomies, n = 4) and were classified as: T1, n = 45; T2, n = 14; T3, n = 5; T4, n = 1. Patients with lymph node invasion were staged as N+. Metastasis was defined according to the clinical findings, if no histopathological data on metastasis were available. In 10 patients, distant metastases were discovered at the time of diagnosis (lung, n = 7; bone, n = 1; brain, n = 1; distant lymph node, n = 1). In 9 patients metastasis occurred after surgery (lung, n = 5; bone, n = 1; inferior vena cava, n = 1; brain n = 1; distant lymph node, n = 1). Fuhrman's nuclear grade was as follows: grade 1, n = 7; grade 2, n = 35; grade 3, n = 19; grade 4, n = 4 (16).

Sample collection

Surgical pieces were immediately analyzed macroscopically. Samples from tumoral areas and from macroscopically normal areas were isolated. Half of them was immediately snap-frozen in liquid nitrogen, the other half was fixed in formaldehyde and further processed for paraffin embedding. A systematic microscopic control of the samples was performed and the tumor samples containing areas of necrosis were not used.

VEGF protein level quantification in whole tissue sections

Sixty whole tissue sections (10 µm thick) were taken from frozen tumoral and non normal renal tissue. Sections were placed in 10 mM Tris-HCL buffer, pH 7.4, containing molybdat and dithiotreithol (Sigma, Saint-Quentin Fallavier, France). Cytosolic extracts from RCC and corresponding normal kidney (n=52) were obtained by centrifugation at 100 000 g for 1 hour at 4°C. VEGF levels were assayed in duplicate by specific sandwich enzyme immunoassay techniques (Quantikine R&D Systems, catalog number DVE00, Minneapolis, MN). The minimum detectable concentration was estimated to be 9 pg/ml. The ELISA test for VEGF recognized the different VEGF isoforms.

Triple Immunofluorescence study

Triple immunofluorescence labellings were performed on 5µm-thick frozen sections from tumoral and non tumoral areas. The sections were incubated with primary antibodies directed against VEGF (mouse anti-human VEGF-A antibody, Santa Cruz biotechnology, Santa Cruz, CA, USA), VEGFR-1 (goat anti-human VEGFR-1, R&D systems, Minneapolis, MN, USA) at 1:100 dilution or VEGFR-2 (goat anti-human VEGFR-2, Santa Cruz, CA, USA) at 1:100 dilution. The sections were then incubated with FITC-conjugated chicken anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution and PE-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:500 dilution. Subsequently, the sections were incubated with mouse cytokeratin antibodies (clone AE1/AE3, at 1:100 dilution, Chemicon, Temecula, CA, USA) which were pre-labelled with Zenon Alexa Fluor 350 mouse IgG labelling kit (Molecular Probes, Eugene, OR, USA). Using an AX 70 Olympus microscope with SIS software, successive pictures were captured on the same area analyzed with different fluorochrome wavelength filters. For each selected area the successive pictures were overlaid. Cells expressing cytokeratins were considered as epithelial cells when cells not expressing cytokeratins were considered as stromal cells.

Combined Laser Microdissection and QRT-PCR

Laser microdissection was performed on epithelial cells and stromal cells on 5µm-thick frozen sections of tumoral (n=33) and non tumoral renal tissue (n=22). On an Olympus BX inverted microscope with PALM laser microdissector, the two different cell populations were successively laser microdissected and catapulted into caps of tubes containing Trizol reagent for RNA extraction. The surface of the different laser microdissected areas was calculated using PALM robot software version 3.0. The systematic quantitative assessment allowed us to laser microdissect a minimum of 500 000 tumor cells and 100 000 stromal cells.

RNA extraction and reverse transcription

RNA was extracted using Trizol reagent (Invitrogen, France). Reverse transcription of total RNA was performed using 200 units of Superscript II RNase H- reverse transcriptase with random hexamers (Invitrogen, France) according to manufacturer's instructions.

Primer and probe design

Quantification of the transcripts coding for $VEGF_{121}$, VEGFR-1 and VEGFR-2 and control gene, β2 microglobulin (B2M) was performed using LightCycler technology (Roche Diagnostics, France). Primers and probes were chosen using Primer Express Software (Applied Biosystems, France) (Table 2). Hydrolyzation probes were labeled with a reporter dye (6-carboxy-fluorescein phosphoramidite) at the 5' end and a quencher dye (5-carboxy-tetramethylrhodamine) at the 3' end.

QRT- PCR

PCR optimization and specificity of RT-PCR products were conducted using SYBR® Green technology, melting curves and agarose gel electrophoresis. Transcript quantification was performed using TaqMan® technology. Quantitative PCR was performed using either the SYBR® Green or LCFastStart DNA Master Mix kit (Roche Diagnostics, France) according to manufacturer's instructions. All experiments were performed in duplicate.

To determine the absolute copy number of the target transcripts, the cDNAs for $VEGF_{121}$ mRNA and its receptors, were cloned in TOPOII TA cloning Kit (Invitrogen, France) following the manufacturer's recommandations. Cloned products were digested with *EcoR* I (Invitrogen Cergy Pontoise, France), extracted from 2% agarose gel, purified with the PCR purification Kit (Qiagen, Courtaboeuf, France). Finally the products were measured in a spectrophotometer, and molecule concentrations were calculated. A Standard curve for each transcript was generated using serial dilutions of cloned products ranging from one or 10 to 10^9 molecules /µl. The copy number of unknown samples was calculated by setting their PCR cycle number (Crossing Point) to the standard curve. To correct for differences in both RNA quality and quantity between samples, the expression levels of interest transcripts were normalized to the housekeeping B2M gene transcripts. Results are presented as copies of target gene per 10^6 copies of B2M.

Statistical analyses

Values for VEGF protein levels and for the gene expression levels of $VEGF_{121}$, VEGFR-1 and VEGFR-2 in tissue extracts are given as median and range. Differences for VEGF protein and mRNA expression between different renal tissue compartments (tumoral and non tumoral, epithelial and stromal) were analyzed by Wilcoxon test for paired data. Univariate analysis of the correlation between the different parameters was done using the non-parametric Spearman rank test. For survival analysis, the stopping date was January 1, 2006. The overall survival (OS) rates were measured from the date of surgery to the study endpoints which were the stopping date, the date of death from RCC, or the time of last visit before the patient was lost to follow up. For the patients free of tumor after surgery, the event-free survival (EFS) was measured from the date of surgery to the study endpoints. Survival rates were estimated by the method of Kaplan and Meier. Univariate analysis was performed using logrank tests. Results for comparison of major endpoints were regarded as significant if the two-sided p was < 0.05. Statistical analysis was performed using the SAS V 913 software package (SAS Institute, Carry, NC).

RESULTS

VEGF protein levels in whole tissue sections

VEGF protein level was significantly higher ($p < 0.0001$) in tumoral tissue than in corresponding non tumoral renal tissue (253 pg/mg, 10-79959 *vs* 45 pg/ml, 10-2491; n=52), (Figure 1). Tumor VEGF protein level was associated with tumor size ($p = 0.03$), tumor grade ($p = 0.02$) and metastasis at diagnosis ($p = 0.006$) (Table 3). Tumor protein levels were significantly higher ($p = 0.01$) in patients with metastasis detected during the follow-up (2061 pg/mg, 78-6179, n = 9) than in patients with no metastasis at all, i.e neither before surgery nor during the follow-up (207 pg/mg, 10-12812, n = 46).

For survival analysis VEGF protein level cut-off value was chosen after quartile analysis, and was the value at the 75th percentile of the values found in tumoral tissue for the 65 patients. The median follow-up was 72 months (range: 58-94 months). Univariate analysis of the survival curves demonstrated the benefit of lower levels of VEGF for event-free survival ($p = 0.02$) (figure 2). This parameter was not an independent prognostic factor in multivariate analysis.

Combined laser microdissection and QRT-PCR according to metastatic and non metastatic status

We have developed for the quantification of each transcript, a highly specific and sensitive QRT-PCR test adapted to small quantities of laser microdissected cells (Figure 3). For all assays, intra- and inter-run variability (calculated from triplicate samples and comparing the results of samples in 10 different runs) showed an average SD for the crossing points of 0.15 and 0.55 respectively. With this combined method the studied transcripts, $VEGF_{121}$ and its receptors VEGFR-1 and -2, have been detected and quantified in all specimens of stromal and epithelial compartments from either tumoral or normal kidney.

Comparing epithelial and stromal cells in tumoral and corresponding non tumoral areas (n=22), $VEGF_{121}$ mRNA was significantly higher in tumoral epithelial ($p = 0.007$) and stromal cells

(p = 0.016) than in corresponding non tumoral cellular populations. Similarly VEGFR-1 mRNA was significantly higher in tumoral epithelial (p = 0.002) and stromal cells (p = 0.003) than in corresponding non tumoral cellular populations (Table 4). No difference in VEGFR-2 mRNA expression was found in laser microdissected epithelial and stromal compartments from tumoral and non tumoral areas. Comparing epithelial and stromal compartments in tumors (n = 33), no significant difference was found either for expression of $VEGF_{121}$, VEGFR-1 or -2. Considering the patients with or without metastasis at diagnosis, no difference was observed regarding $VEGF_{121}$, VEGFR-1 and -2 expressions. In this group of 33 patients with RCC analyzed with combined laser microdissection and QRT-PCR, no association of $VEGF_{121}$, R1 and R2 mRNA values were found with the stage of the disease or survival.

Immunofluorescent labelling of VEGF, VEGFR-1, VEGFR-2 and cytokeratins

In tumoral areas (Figure 4), VEGF was strongly expressed by tumor cells and by some stromal cells. Coexpression of VEGF and VEGFR-1 was observed in tumor cells and interestingly in stromal cells of tumoral areas. No cell expressed VEGFR-1 alone. Around 1 of 10 cytokeratin positive cells coexpressed VEGF and VEGFR-1. In corresponding non tumoral tissue VEGF was seldom expressed. It was not found in glomerular or vascular areas, but in rare tubular sections. In non tumoral kidney, no coexpression of VEGF and VEGFR-1 was observed and no stromal cell was labeled. In tumoral and non tumoral areas (Figure 5), no cell expressed VEGFR-2.

DISCUSSION

In this study of 65 patients with RCC, with an 8 year follow-up, we have shown that VEGF protein levels in tumor cytosolic extracts were associated with tumor progression. VEGF protein level measure in RCC cytosolic extracts is an easy-to-perform and reliable test. Several groups have analyzed VEGF expression in RCC at the mRNA and/or at the protein level using immunohistochemistry or western blot (17-21), but assessment of VEGF protein level in RCC tumor extracts has not been reported. The two quantitative techniques that could be used in clinical situations are QRT-PCR at the mRNA level and ELISA in cytosolic extracts at the protein level. In other types of cancer, particularly in breast cancer (22, 23), VEGF tumor content measured in cytosolic extracts has been shown to have a prognostic significance.

Considering mRNA expression, different groups have reported an increase in VEGF mRNA expression in RCC, compared with normal renal tissue (24, 25). A higher tumor VEGF mRNA expression has been associated with a worse prognosis in different series of RCC (20, 21). As for VEGF isoforms a predominant expression of $VEGF_{121}$ and $VEGF_{165}$ transcripts has been reported in different series of RCC (25-27), together with a higher VEGFR-1 and R-2 mRNA level in RCC compared to normal kidney in one series (27). We have extended these results, using laser microdissection coupled with QRT-PCR, with the analyses of VEGF and its receptors expression in microdissected RCC epithelial and stromal compartments. When compared to corresponding non tumoral renal cells, $VEGF_{121}$ and VEGFR-1, but not VEGFR-2, mRNA levels were higher in RCC epithelial and stromal cells. This suggests that epithelial as well as stromal cells contribute to the increased VEGF and VEGFR-1 expression in RCC. Along the same line, experimental data in a xenograft model of breast cancer demonstrate that the tumor growth inhibition is the strongest when human VEGFR-1, expressed by the tumor cells, and murine VEGFR-1, expressed by the stromal cells, are concomitantly inhibited (28).

To further characterize the type of cell expression of VEGF and VEGFR-1, we used a triple immunofluorescence labeling on whole tissue sections. Interestingly, we observed a colocalization

of VEGF and VEGFR-1 on some RCC tumor epithelial and stromal cells. Such a coexpression was not observed in any type of cells in normal kidney.

Taking together, these results are in accordance with recent studies demonstrating a major role for VEGFR-1 signaling in tumor progression (28-32). VEGFR-1 participates in the migration of neoplastic epithelial cells (11, 32) as of several others types of non-endothelial cells, such as hematopoietic stem cells and monocytes. For leukemic cells it has been shown that VEGFR-1 is essential for tumor cell growth via a VEGF/VEGFR-1 autocrine loop (33, 34). During progression of solid tumors, hematopoietic progenitor cells expressing VEGFR-1 might initiate the pre-metastatic niche (31). The demonstration, in a murine model of RCC, of the inhibition of lung metastasis through administration of a soluble form of VEGFR-1 also underlines the role of VEGFR-1 in RCC progression (30).

We have demonstrated that in RCC both epithelial and stromal cells contribute to VEGF and VEGFR-1 overexpression, and that VEGF overexpression is linked to tumor progression. These results contribute to a better knowledge of cell targets of anti-VEGF therapies. Along this line, further studies are needed to establish the value of VEGF protein assay in RCC cytosolic extracts for the patients who could benefit of anti-VEGF therapies.

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Table 1: Characteristics of patients

Patients	**N= 65**
Median Age, Range	65, 31-88
Gender	
Male	47
Female	18
Nephrectomy	
Radical	59
Partial	2
Tumorectomy	4
TNM	
Size (pT)	
T1	45
T2	14
T3	5
T4	1
Nodes (pN)	
N+	4
N-	61
Metastasis	
M+	10
M-	55
Furhman's Grade	
n 1	7
n 2	35
n 3	19
n 4	4

Table 2: Specific primer and probe sequences, used for real-time QRT-PCR.

Name	Sequence	Exon junction	Product size (bp)
VEGF $_{121}$ - sense	5'-gAgCTTCCTACAgCACAACAAA-3'	5-8	99
VEGF $_{121}$ - antisense	5'-CTCggCTTgTCACATTTTTC-3'		
VEGF $_{121}$ - probe	5'-TgCAgACCAAAgAAAgATAgAgCAAgACA		
VEGFR-1- sens	5'-CgACgTgTggTCTTACggAgTA-3'	24-25	107
VEGFR-1- antisense	5'-CTTCCCTCAggCgACTgC-3'		
VEGFR-1- probe	5'-TgTgggAAATCTTCTCCTTAggTgggTCTC-3'		
VEGFR-2- sens	5'-TCTCAATgTggTCAACCTTCTAgg-3'	19-20	79
VEGFR-2- antisense	5'-AAATTTgCAgAATTCCACAATCAC-3'		
VEGFR-2- probe	5'-TgTACCAAgCCAggAgggCCACTC-3'		
B2M- sens	5'CgCTCCgTggCCTTAgC 3'	1-2	70
B2M- antisense	5' gAgTACgCTggATAgCCTCCA 3'		
B2M- probe	5'TgCTCgCgCTACTCTCTCTTTCTggC 3'		

Table 3. VEGF (pg/mg) in RCC tissue according to histopathology, staging and grading

Size		**Grade**		**Metastasis at diagnosis**	
<7 cm (45)*	≥7cm (20)	Low† (42)	High (23)	M0 (46)	M (10)
241 (10 - 79959)	413 (14 - 12812)	164 (14 - 6087)	470 (10 - 79959)	207 (10 - 12812)	1516 (86 - 79959)
p = 0.03		**p = 0.02**		**p = 0.006**	

* The numbers in parenthesis represent the number of patients in each group
† Low grade is grade 1 - 2 and high grade is grade 3 - 4
‡ Results are given as median (range)
§ Wilcoxon test, $p < 0.05$ was considered as statistically significant

Table 4: **$VEGF_{121}$, VEGFR-1 and -2 transcript expression in laser microdissected compartments (epithelium and stroma) of normal and tumoral renal tissue according to the metastatic status.**

		Whole population (n = 22)	M0* (n = 12) †	M+* (n = 10)
TE* *vs* NTE*	**$VEGF_{121}$**	4.8 (0-125) *vs* 2.6 (0-8.6), **p = 0.007**	4.3 (0-125.0) *vs* 2.5 (0.1-8.6) ‡, **p = 0.03**	6.0 (1.6- 42.0) *vs* 3.5 (0- 5.6), p = 0.11 §
	VEGFR-1	2.9 (0.1-197.5) *vs* 0.5 (0-10.2), **p = 0.002**	2.0 (0.05-197.5) *vs* 0.6 (0-3.4), **p = 0.02**	4.3 (0.4- 68.6) *vs* 0.5 (0- 10.3), **p = 0.03**
	VEGFR-2	9.5 (0- 104) *vs* 8.3 (0- 41.4), p = 0.51	11.4 (0.2-104) *vs* 10.2 (0- 41.4), p > 0.99	9.5 (0- 54.3) *vs* 2.8 (0.3- 27.3), p = 0.14
TS* *vs* NTS*	**$VEGF_{121}$**	6.9 (0.6-165.0) *vs* 1.0 (0-23.2), **p = 0.02**	8.0 (0.9-165.0) *vs* 0.5 (0-15.2), **p =0.02**	6.9 (0.6- 31.1) *vs* 2.6 (0- 23.2), p = 0.40
	VEGFR-1	5.3 (0-435.0) *vs* 0.5 (0-54.7), **p = 0.003**	4.5 (0- 435) *vs* 0.3 (0- 54.7), p = 0.12	7.8 (0-108.7) *vs* 0.6 (0- 4.7), **p = 0.007**
	VEGFR-2	6.5 (0- 132.3) *vs* 1.3 (0- 76.9), p = 0.33	6.5 (0- 54) *vs* 1.3 (0- 76.9), p = 0.87	9.8 (0- 132.3) *vs* 1.3 (0- 65.7), p = 0.14

* M0: non metastatic, M+ metastatic, TE: tumoral epithelial cells,
NTE: non tumoral epithelial cells, TS: tumoral stromal cells, NTS: non tumoral stromal cells,
† The numbers in parenthesis represent the number of patients in each group
‡ Results are given as median (range)
§ Wilcoxon test for paired data, $p < 0.05$ was considered as statistically significant.

LEGENDS TO FIGURES

Figure 1

Individual values of VEGF (pg/mg) in RCC and normal tissue. Data points, mean of two determinations, bars, median values; p value was calculated using the nonparametric Wilcoxon test for paired data.

Figure 2

Event-free-survival after surgery was estimated according to the VEGF levels in tumor extracts. The cut-off value for VEGF is the 75th percentile of VEGF levels in the tumor; p value was calculated using the logrank test.

Figure 3

$VEGF_{121}$, VEGFR-1 and VEGFR-2 mRNA calibration curves and agarose gel electrophoresis TaqMan probe PCR products showing specificity of real-time RT-PCR. V9, V8, V7, V6, V5, V4, V3, V2, V1 and one correspond respectively to: 10^9, 10^8, 10^7, 10^6,10^5,10^4,10^3,10^2, 10 and one copies of transcripts. For all the assays, intra- and inter-run variability (calculated from triplicate samples and comparing the results of samples in 10 different runs) showed an average SD for the crossing points of 0.15 and 0.55 respectively. The DNA marker used on agarose gel electrophoresis was pUC19 DNA/Msp1 (HpaII) Marker, 23 (Fermentas, France).

Figure 4

Expression of VEGF, VEGFR-1 and cytokeratin (AE1/AE3) in RCC (A and B) and in non tumoral areas (C). Triple immunofluorescence labelling of VEGF-R1, VEGF, and cytokeratins showed that cytokeratin-positive RCC cells coexpressed VEGF and VEGFR-1

(epithelial cells surrounded by broken lines in A and B); cytokeratin negative round (A) or fusiform (B) cells coexpressed also VEGF and VEGFR-1 (arrows). In the normal kidney, no cell whether tubular (T), glomerular (G) or interstitial expressed VEGFR-1. Few tubular cells expressed slightly VEGF. Scale bars = 10 µm.

Figure 5

Expression of VEGF, VEGFR-2 and cytokeratin (AE1/AE3) in RCC (A) and in non tumoral areas (B). Identical methods of triple immunofluorescence labelling for VEGFR-2, VEGF, and cytokeratins did not show any coexpression of VEGF and VEGFR-2 whether in RCC (A) or in non tumoral renal tissue (B). T: tubular area, G: glomerular area. Scale bars = 10 µm.

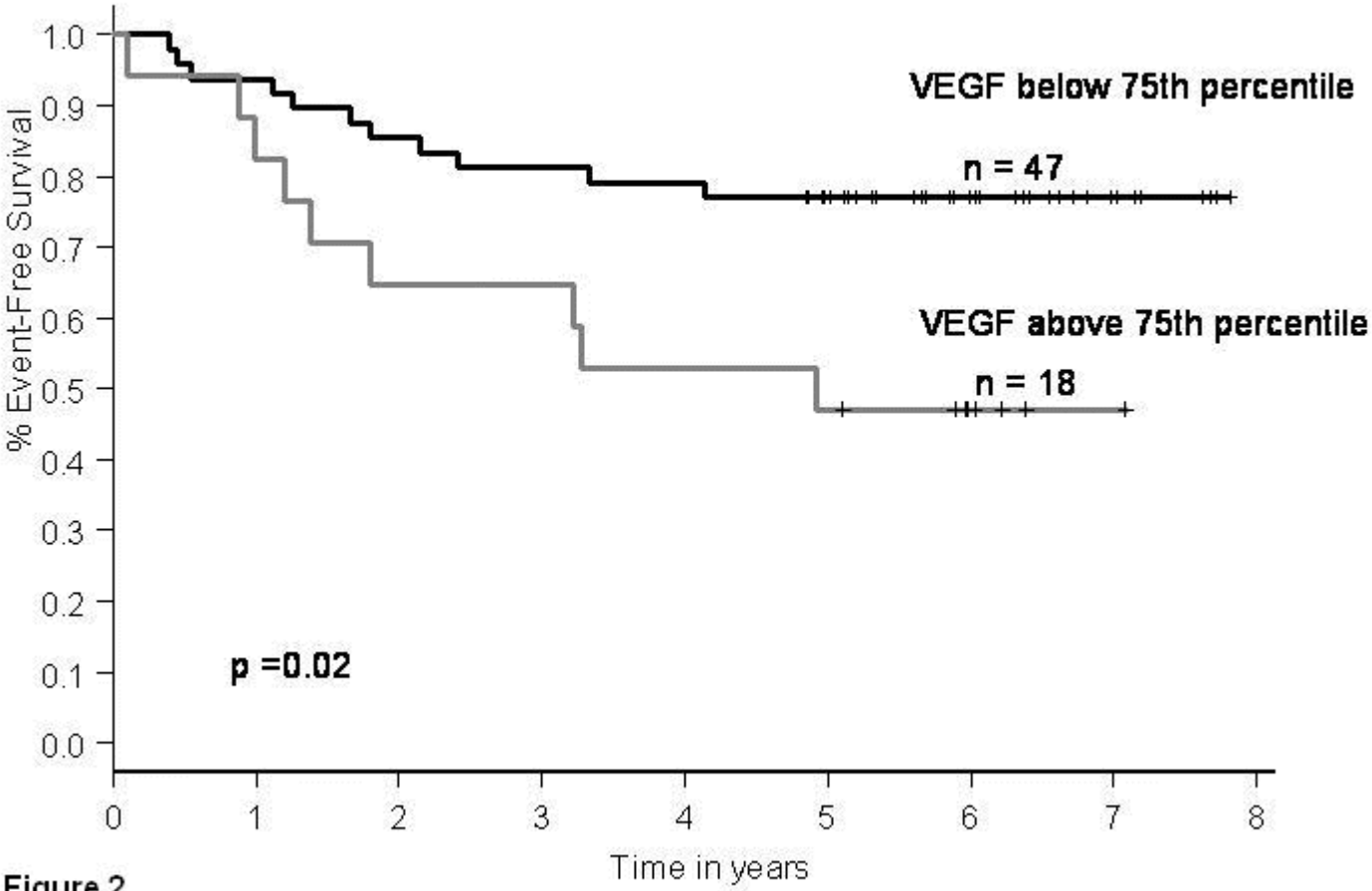
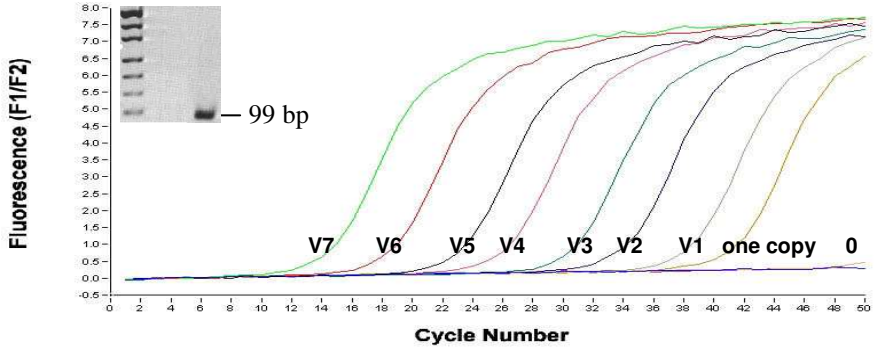
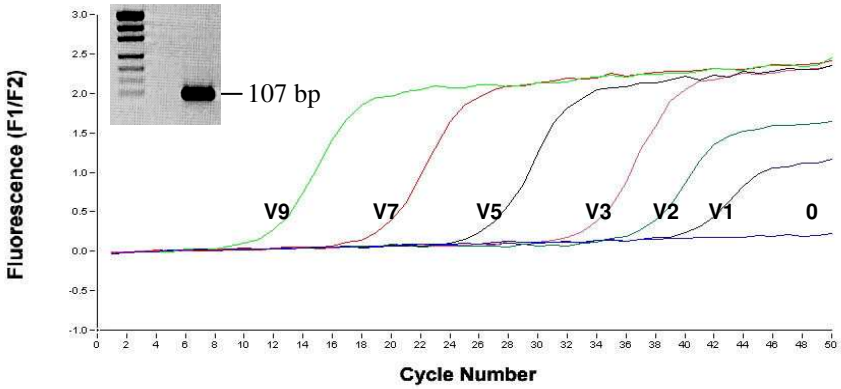


Figure 2

VEGF $_{121}$



VEGFR-1



VEGFR-2

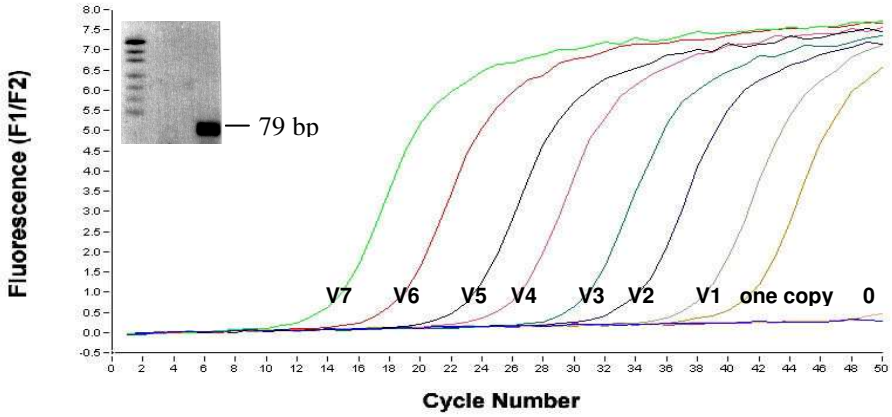
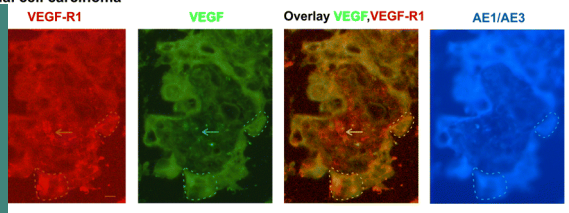
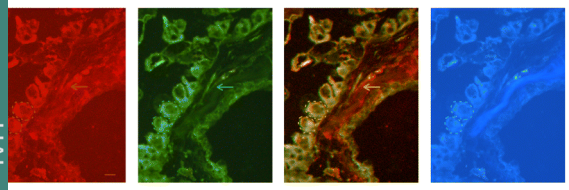


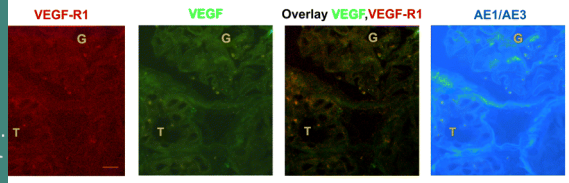
Figure 3

Renal cell carcinoma





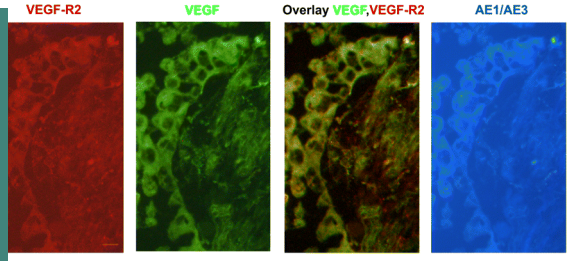
tumoral renal tissue



ure 4

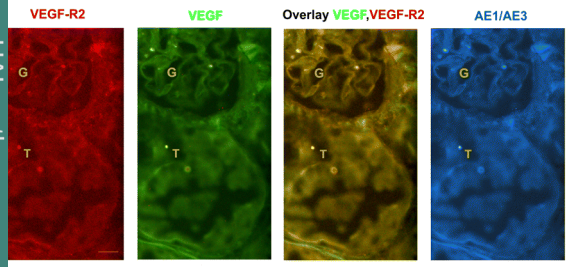
Renal cell carcinoma

A



N tumoral renal tissue

B



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